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Matrix metalloproteinase inhibitors prevent sepsis-induced refractoriness to vasoconstrictors in the cecal ligation and puncture model in rats

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ABSTRACT

Previous studies have shown that the loss of contractility in aortas from lipopolysaccharide (LPS)-treated rats is related to intracellular activation of matrix metalloproteinase (MMPs). However, the role of MMPs in the vascular refractoriness to vasoconstrictors has not been investigated in a model of polymicrobial sepsis. We evaluated the effects of the oral administration of the MMP inhibitors doxycycline or ONO-4817 in the *in vitro* vascular reactivity of aortic rings from rats subjected to the cecal ligation and puncture (CLP) model of sepsis. Both doxycycline and ONO-4817 did not change vascular responses in sham-operated rats, but fully prevented hyporeactivity to KCl, phenylephrine and angiotensin II in vessels from CLP rats. This protective effect was not associated with changes in hematological parameters or blood nitrate and nitrite. The refractoriness to contractile agents was accompanied by enhanced activity of MMP-2 in aorta from CLP rats, which was abrogated by MMP inhibitors. CLP-induced sepsis did not impair the levels of MMP-2 in aorta, but significantly reduced calponin-1, a regulatory protein of vascular contraction. In addition, augmented levels of TIMP-1 were found in vessels from CLP rats. All these differences were prevented by either doxycycline or ONO-4817. Our study shows, for the first time in the CLP rat model of sepsis, that the vascular refractoriness to different contractile agents induced by polymicrobial sepsis is associated with increased activity of MMP-2 and reduced amounts of calponin-1 in the aorta. These findings reinforce the importance of the enhanced activity of MMPs for vascular failure in septic shock.

1. Introduction

Matrix metalloproteinases (MMPs) were described in vertebrates in early 1960s (Gross and Lapiere, 1962) and had their role in the remodeling of extracellular matrix extensively investigated. The wide distribution of MMPs in different cell types, including human monocytes, neutrophils, smooth muscle cells and endothelial cells, stimulated investigations to characterize the involvement of the proteolytic activity of MMPs in several biological processes. The participation of MMPs in human inflammatory conditions, such as arthritis (Okada et al., 1989), periodontal disease (Golub et al., 1997), and atherosclerosis (Galis et al., 1994), is now well characterized (for review see Castro et al., 2011; Hu et al., 2007; Parks et al., 2004). The attention for the involvement of MMPs in sepsis was raised by the demonstration of high levels of MMP-9 in the blood of non-surviving septic patients, which was associated with increased amounts of monocyte MMP-9 mRNA and was reduced after removal of bacterial lipopolysaccharide (LPS) from blood (Nakamura et al., 1998). Subsequent studies reinforced the ability of LPS and several other inflammatory mediators to induce an *in vitro* rapid release of MMP-9 in whole human blood (Pugin et al., 1999), comparable to the release of MMP-9 in the blood of healthy humans after LPS injection (Albert et al., 2003; Pugin et al., 1999). Inhibition of MMPs *in vivo* prevented the lung injury (Carney et al., 2001) and protected against the lethality associated with endotoxemia (Hu et al., 2005) and with the cecal ligation and puncture (CLP) model of sepsis in rats (Steinberg et al., 2003).

Severe sepsis and septic shock involve an exacerbated inflammatory process and are accompanied by a progressive reduction in blood pressure, which is refractory to fluid infusion and

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vasoactive drugs and limits blood supply to peripheral and vital organs, contributing to multiple organ failure and the high rate of sepsis-associated mortality (for review see Dellinger, 2003). Interestingly, the inhibition of MMPs in both *in vitro* (Lalu et al., 2006) and *in vivo* (Cena et al., 2010) approaches prevented LPS-induced hyporeactivity to vasoconstrictors, and the MMP-2 activity was augmented in homogenates of aorta taken from endotoxemic rats, in a manner fully dependent on the presence of endothelial cells and nitric oxide production (Cena et al., 2008). Nevertheless, if on one hand high blood levels of active MMPs have been clearly associated with reduced survival in both experimental and clinical studies, on the other hand the evidence of their involvement in vascular dysfunction are restrict to the demonstration of a role for MMP-2 in the hyporeactivity to vasoconstrictors in vessels from LPS-injected rats (Castro et al., 2012; Cena et al., 2010). Importantly, although well accepted as an experimental model of sepsis, endotoxemia does not reproduce several clinical aspects of sepsis (Buras et al., 2005; Dejager et al., 2011; Rittirsch et al., 2009).

In this light, this study was designed to investigate if MMPs also play a role in the development of vasoplegia during the polymicrobial sepsis induced by CLP, an experimental model that closely resembles the clinical progression and characteristics of human septic shock.

2. Material and methods

2.1. Drugs and reagents

N-[(1S,3S)-1-[(Ethoxymethoxy)methyl]-4-(hydroxyamino)-3-methyl-4-oxobutyl]-4-phenoxybenzamide (ONO-4817; a kind gift from ONO Pharmaceutical, Osaka, Japan); doxycycline, acetylcholine, phenylephrine, angiotensin II, gelatin type A from porcine skin, Coomassie brilliant blue (G-250), Tris–HCl, sucrose, leupeptin, soybean trypsin inhibitor, aprotinin, protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON, CA); dithiothreitol (Fisher Scientific, Ottawa, ON, CA); antibodies against β-actin, calponin-1, smoothelin-B (Santa Cruz, Dallas, TX, USA), MMP-2 (Millipore, Mississauga, ON, CA), TIMP-1 (Calbiochem, EMD Millipore, Mississauga, ON, CA) and TIMP-4 (Chemicon, Temecula, CA, USA); Triton X-100 (Pierce, Thermo Scientific, Rockford, IL, USA); NaCl, $CaCl_2$, NaN_3, KCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4$, KH_2PO_4, $NaHCO_3$, d-glucose 11.1 (Merck & Co, EMD Millipore, Mississauga, ON, CA).

2.2. Animals, experimental groups and treatment with matrix metalloproteinase inhibitors

Male Wistar rats (250–300 g) supplied by the University of Alberta were used in the experiments. All procedures were approved by the Institutional Animal Care and Use Committee of University of Alberta and were in compliance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Two hours prior to surgery, animals used in both CLP and sham-operated groups ($n=4$–8 per group) received a single gavage containing the MMP inhibitors ONO-4817 (100 mg/kg; Hariya et al., 2004), doxycycline (15 mg/kg; Yaras et al., 2008), or 0.5% carboxymethylcellulose (0.1 ml/100 g), the vehicle used to dilute either of the MMP inhibitors. For comparison purposes, all experiments were also performed in a group of naïve rats ($n=4$).

2.3. Induction of sepsis by CLP

The CLP model was conducted as previously described (Rittirsch et al., 2009) with minor modifications. Briefly, the rats were anesthetized with ketamine/xylazine (100/20 mg/kg, i.p.) and a skin midline incision was performed after disinfection of the abdominal area. The cecum was ligated distal to the ileocecal valve (comprising 75%) and the antimesenteric cecal surface was punctured with a needle (14 gauge, one hole), followed by a mild compression for leakage of the intestinal content. The wound was closed by interrupted sutures. After the surgery, all animals received a postoperative fluid resuscitation (sterile saline, 3 ml/100 g, s.c.) to prevent dehydration, and buprenorphine (0.01 mg/kg) to relieve pain. The animals were maintained in warmed cages (~35 °C) until the complete recovery of anesthesia. Sham-operated animals were subjected for the same procedures, with the exception of the ligature or intestinal perforation.

2.4. Experimental setup for in vitro assessment of vascular responsiveness

Six hours after the surgical procedure, the rats from CLP, sham-operated or naïve groups were euthanized using an overdose of sodium pentobarbital (100 mg/kg, i.p.). The thorax was opened and the descending thoracic aorta was removed and cleaned of connective tissue. The distal portion was immediately frozen in liquid nitrogen and stored at −70 °C for biochemical analysis. The proximal portion was cut into 3–4 mm ring-like segments and mounted in tissue bath chambers containing Krebs solution (composition, in mM: NaCl 115.3, KCl 4.9, $CaCl_2 \cdot 2H_2O$ 1.46, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, d-glucose 11.1; pH 7.4), maintained at 37 °C and continuously bubbled with 95% O_2/5% CO_2 gas mixture. The tension was recorded *via* isometric force transducers (Grass FT03) connected to a MP System (model MP150) coupled to Acq-Knowledge 3.1 recording software (both from Biopac Systems, Goleta, CA, USA).

The aortic rings were allowed to stabilize under a resting tension of 1.0 g for 60 min before the addition of any drug. During all stabilization periods followed in our protocols the Krebs solution was replaced every 15 min. After stabilization, the aortic rings were exposed to 60 mM KCl and the contraction obtained was recorded. The preparations were washed with Krebs solution and after a new stabilization period of 30 min, phenylephrine, followed by acetylcholine (both 1 μM), were added into the baths. The ability of acetylcholine to induce at least 80% relaxation in phenylephrine-contracted preparations was used to confirm the integrity of their endothelium. Only endothelium-intact aortic rings were used in this study.

After washing and following a new resting period of 60 min, cumulative concentration response curves to phenylephrine (1 nM to 3 μM) or angiotensin II (1 nM to 0.3 μM) were constructed. Each preparation was exposed to only one of these vasoconstrictors. The contractile effects of these vasoconstrictors were compared among the groups.

2.5. Collection of blood, measurement of serum levels of nitrate and nitrite and hematological analysis

Samples of blood were taken from the vena cava immediately before the isolation of the thoracic aorta. Hematological parameters were evaluated in 10 μl of fresh blood collected from control and CLP groups, with or without previous treatment with doxycycline, using an automatized blood counter (HORIBA ABX®, Micros 60; Montpellier, France). The remaining blood was centrifuged at 7500 g for separation of serum, which was collected and frozen at −70 °C. The serum was processed for the colorimetric detection of nitrate and nitrite (NO_x^-) using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer's instructions.

Table 1
The reduction in the maximal contractile effect (E_{max}) in aortic rings at 6 h after CLP surgery is prevented by MMP inhibitors.

	Naïve	Sham-operated			CLP		
	–	Vehicle	ONO-4817	Doxycycline	Vehicle	ONO-4817	Doxycycline
KCl [60 mM]	2.52 ± 0.24	2.14 ± 0.28	2.23 ± 0.28	2.27 ± 0.26	1.36 ± 0.11[a]	2.09 ± 0.16[b]	2.04 ± 0.09[b]
Phenylephrine [1 μM]	2.29 ± 0.29	2.15 ± 0.27	1.97 ± 0.24	2.01 ± 0.24	1.54 ± 0.19[a]	2.06 ± 0.26[b]	2.02 ± 0.25[b]
Angiotensin II [0.3 μM]	1.09 ± 0.17	0.88 ± 0.14	0.81 ± 0.10	0.82 ± 0.12	0.28 ± 0.04[a]	0.79 ± 0.13[b]	0.68 ± 0.12[b]

The values show the mean ± standard error of the maximal contractile effects (E_{max}, in grams) obtained in vessels from 4–8 different animals per group. Sham-operated and CLP rats were previously treated with ONO-4817 (100 mg/kg, p.o.), doxycycline (15 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose, 0.1 ml/100 g), two hours before the CLP or sham surgery.

[a] $P < 0.05$ *versus* control (sham-operated) group.
[b] $P < 0.05$ when compared with the CLP-vehicle group.

2.6. Western blot and zymography analysis

For this assay the frozen aortas (−70 °C) were crushed in liquid nitrogen and homogenized on ice using a pellet pestle hand homogenizer in a buffer containing Tris–HCl (50 mmol/L), sucrose (3.1 mmol/L), dithiothreitol (1 mmol/L), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), aprotinin (2 μg/ml), protease inhibitor cocktail (1:1000 v/v), and Triton X-100 (0.1%). After homogenization, the samples were centrifuged at 10,000 g (20 min, 4 °C), and the supernatants were collected for analysis. The total protein concentration in the samples was determined by the bicinchoninic acid assay, using a commercial kit (Sigma-Aldrich, Oakville, ON, CA) and bovine serum albumin as standard.

Western blot analyses on the aortic extracts were performed as previously detailed (Guarido et al., 2014). In brief, samples containing 40 μg of protein were subjected for electrophoretic separation in 10% polyacrylamide gel (SDS-PAGE), transferred onto a polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA) in Towbin buffer (20% v/v methanol, 25 mM Tris-base, 192 mM glycine, 0.05% *w/v* sodium dodecyl sulfate), which were exposed overnight to primary antibodies against β-actin (1:25000), calponin-1 (1:500), smoothelin-B (1:500), MMP-2 (1:1000), or TIMP-1 (1:500), followed by incubation with secondary antibodies (1:5000) conjugated to horseradish peroxidase at room temperature for one hour. Enhanced chemiluminescence was used for detection of protein levels by autoradiography, and the bands were quantified by densitometry using the software ImageJ (NIH, USA).

Aliquots containing 20 μg of protein taken from the same aorta homogenate prepared for Western blot were used to measure the gelatinolytic activity of MMPs by zymography using 8% polyacrylamide gels copolymerized with gelatin (2 mg/ml), a substrate of MMP-2. The supernatant of phorbol ester activated HT-1080 cells (American Type Culture Collection) was loaded and used as an internal standard in all experiments. Following 90 min of electrophoresis (at 90 V), the gels were washed with 2.5% Triton X-100 for one hour at room temperature (with 20 min solution changing intervals) to remove sodium dodecyl sulfate. The gels were then maintained at 37 °C during 20 h in the incubation buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3) followed by staining with 0.05% Coomassie brilliant blue (G-250) in a mixture of methanol, acetic acid and water (in the ration 2.5:1:6.5, v/v, respectively) for 2 h and destained in aqueous solution of 4% methanol and 8% acetic acid (v/v) until the transparent bands were detected against the dark blue background. The bands were quantified by densitometry using the software ImageJ (NIH, USA) and expressed as a ratio of the internal standard.

2.7. Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined using two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. A *P* value less than 0.05 was considered statistically significant. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. MMP inhibitors prevent CLP-induced vascular refractoriness without change NO_x^- levels and blood cell count in septic rats

When compared with naïve and sham-operated animals, the aortic rings from rats subjected to CLP showed marked hyporeactivity to vasoconstrictors ($P < 0.05$, compared with the sham-operated group), as shown by a reduction in the maximal contractile effect (E_{max}) induced by phenylephrine, angiotensin II, and KCl (Table 1). The pretreatment with the MMP inhibitors doxycycline or ONO-4817 did not cause any change in E_{max} (Table 1), nor in the concentration-response curves to phenylephrine and angiotensin II in aortic rings from sham-operated animals (Fig. 1A and C). However, the single oral treatment with either doxycycline or ONO-4817 prevented CLP-induced hyporeactivity to KCl, phenylephrine and angiotensin II (Table 1; Fig. 1B and D) ($P < 0.05$ when compared with the CLP-vehicle group). Notably, the comparison of the mean differences between our two independent variables (sepsis and MMP inhibitors) by means of two-way ANOVA revealed a consistent interaction between these factors, which were of borderline of statistical significance for angiotensin II ($P = 0.0569$), and statistically significant for phenylephrine ($P = 0.0385$), confirming that the ability of MMP inhibitors to increase the reactivity for these two vasoactive agents was dependent on the septic state.

High amounts of NO_x^-, low leukocytes, lymphocytes and platelets, as well as increased granulocytes, hemoglobin and hematocrit were found in the blood of rats subjected to CLP, compared with control ($P < 0.05$). Despite the protective effect of doxycycline and ONO-4817 against the vascular hyporeactivity to vasoconstrictors, inhibition of MMP did not influence the augmented blood levels of NO_x^- (Fig. 2) nor the hematological impairment found in the CLP group (Table 2).

3.2. Effects of CLP-induced sepsis on the activity and levels of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in aorta homogenates

Using gelatin zymography we found augmented activity of MMP-2 in aorta from the CLP group, compared with the untreated, sham-operated animals ($P < 0.05$). Doxycycline or ONO-4817 treatment did not alter the activity of MMP-2 in sham-operated animals, but prevented its increase ($P < 0.05$) in aortas from animals subjected to CLP (Fig. 3). As found for vascular reactivity, the

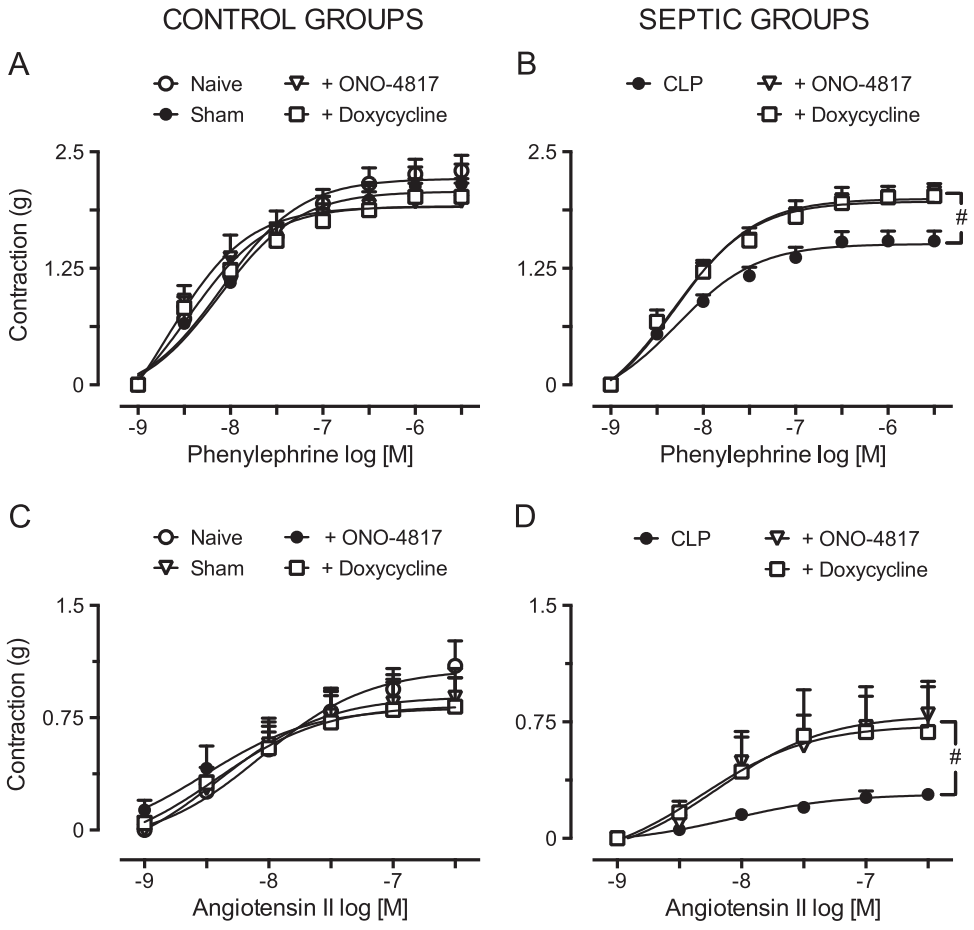

Fig. 1. Matrix metalloproteinase inhibitors prevent vascular refractoriness to vasoconstrictors 6 h after CLP-induced sepsis. The contractile effects induced by phenylephrine and angiotensin II were measured in aortic rings from naïve and sham-operated rats (A and C), and animals subjected to the CLP model of sepsis (B and D), with or without the previous oral administration of ONO-4817 (100 mg/kg) or doxycycline (15 mg/kg), given two hours before CLP or sham surgery. The results show the mean ± S.E.M of 4–6 animals per group. $^{*}P < 0.05$ *versus* the respective control (sham-operated) group in panel A or C; $\#P < 0.05$ when either CLP-ONO-4817 or CPL-Doxycycline groups were compared with the CLP-vehicle group.

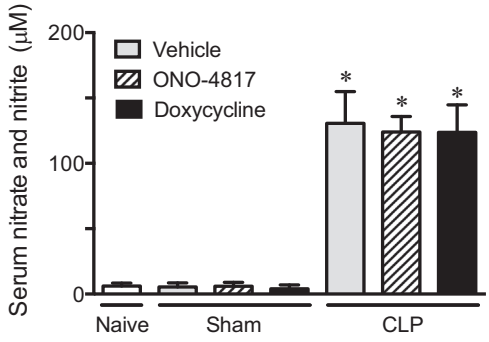

Fig. 2. Lack of effects of MMP inhibitors on serum nitrate and nitrite concentrations measured at 6 h after CLP-induced sepsis. The levels of nitrate and nitrite were detected in serum samples from naïve, sham-operated, or CLP rats previously treated with ONO-4817 (100 mg/kg, p.o.), doxycycline (15 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose, 0.1 ml/100 g), given two hours before CLP or sham surgery. The results show the mean ± S.E.M of samples from 4–6 animals per group. $^{*}P < 0.05$ *versus* sham-operated group.

Table 2
Lack of effects of doxycycline in the hematological changes induced by sepsis in the CLP model.

	Control	CLP	
	–	Vehicle	Doxycycline
Leukocytes (10^3/μl)	11.13 ± 1.04	7.12 ± 0.67[a]	8.05 ± 0.9[a]
Hemoglobine (10^3/μl)	11.94 ± 0.33	16.11 ± 0.43[a]	13.85 ± 0.76[a]
Hematocrit (%)	44.94 ± 1.37	58.73 ± 0.84[a]	52.98 ± 2.89[a]
Platelets (10^3/μl)	850.60 ± 28.26	715.40 ± 51.84[a]	561.80 ± 125.70[a]
Lymphocytes (10^3/μl)	8.32 ± 0.82	3.28 ± 0.32[a]	3.86 ± 0.56[a]
Monocytes (10^3/μl)	1.07 ± 0.07	0.92 ± 0.06	1.13 ± 0.26
Granulocytes (10^3/μl)	1.72 ± 0.24	2.91 ± 0.33[a]	3.20 ± 0.34[a]

The values show the mean ± standard error mean of samples collected from 6 different animals per group. CLP rats were previously treated with doxycycline (15 mg/kg, p.o.), or vehicle (0.5%carboxymethylcellulose, 0.1 ml/100 g), two hours before the CLP surgery.

[a] $P < 0.05$ *versus* control group.

interaction factor for sepsis and MMP inhibitors was also significant for MMP-2 activity ($P=0.0086$).

Western blot analysis revealed that the levels of MMP-2 protein remain unchanged in aortas from the CLP group, and was not affected by doxycycline or ONO-4817 in any experimental group (Fig. 4A). In addition, we also detected increased levels of TIMP-1 in homogenates from CLP animals, compared with sham-operated animals ($P < 0.05$). The treatment with ONO-4817 or doxycycline did not change the protein levels of TIMP-1 detected in aortas from sham-operated and CLP groups (Fig. 4B).

3.3. MMP inhibitors restore calponin-1 but not smoothelin-B levels in the aorta of septic rats

Despite the absence of changes in the expression of smoothelin-B (Fig. 5A), the level of calponin-1 was reduced by 50% in

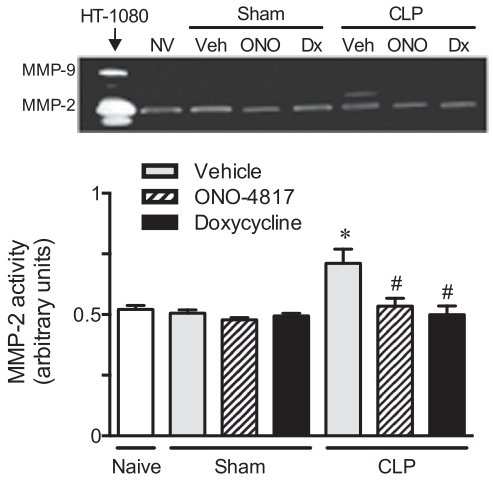

Fig. 3. MMP inhibitors prevent the enhanced activity of MMP-2 in aorta of rats at 6 h after CLP-induced sepsis. Gelatinolytic activity of MMP-2 in aorta homogenates from naïve (NV), sham-operated, or CLP rats treated with ONO-4817 (ONO; 100 mg/kg, p.o.), doxycycline (Dx; 15 mg/kg, p.o.), or vehicle (Veh; 0.5% carboxymethylcellulose, 0.1 ml/100 g), two hours before CLP or sham surgery. The bands were quantified as a ratio of the internal standard control (HT-1080), as indicated in the representative experiment. The results show the mean ± S.E.M of 3–5 experiments with samples from different animals per group. $^{*}P < 0.05$ *versus* control (sham-operated) group, and $\#P < 0.05$ when compared with the CLP-vehicle group.

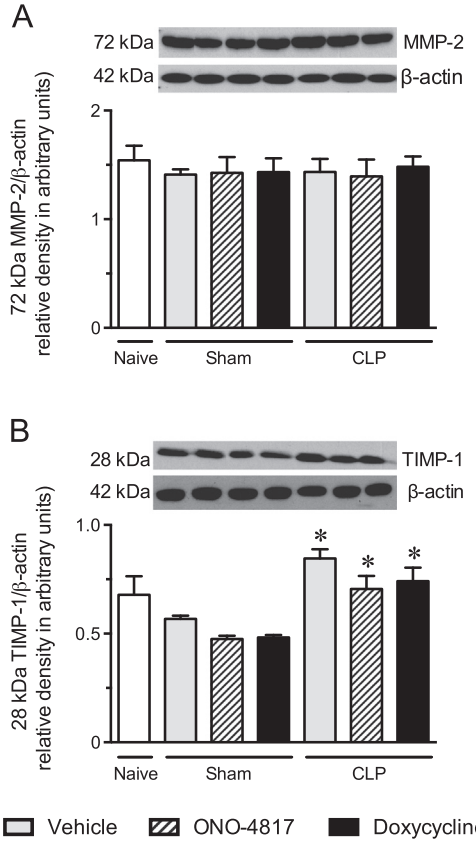

Fig. 4. Western blot analyses for MMP-2 and TIMP-1 in aortas at 6 h after CLP-induced sepsis. The 72 kDa MMP-2 (A) and 28 kDa TIMP-1 (B) were measured in aorta homogenates from naïve, sham-operated, or CLP rats treated with ONO-4817 (100 mg/kg, p.o.), doxycycline (15 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose, 0.1 ml/100 g), at two hours before the CLP or sham surgery. The results show the mean ± S.E.M of 3–4 experiments with samples from different animals per group. $^{*}P < 0.05$ *versus* control (sham-operated) group.

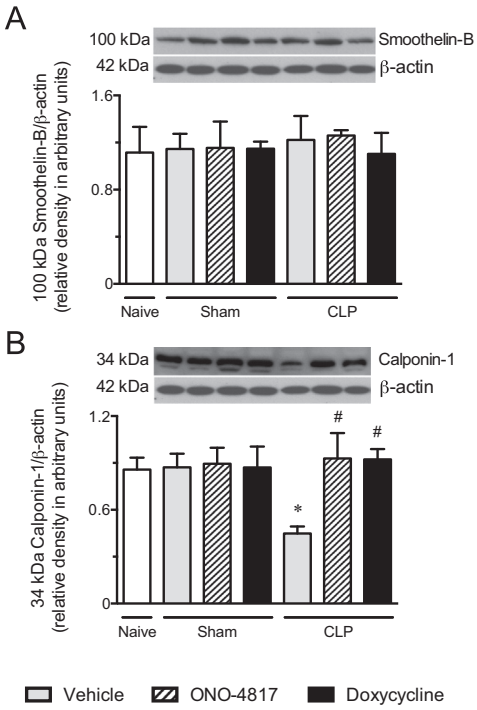

Fig. 5. Prevention of loss of calponin-1, but not smoothelin-B, by MMP inhibitors in aortas at 6 h after CLP-induced sepsis. The 100 kDa smoothelin-B (A) and the 34 kDa calponin-1 (B) were detected in aorta homogenates from naïve, sham-operated, or CLP rats treated with ONO-4817 (100 mg/kg, p.o.), doxycycline (15 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose, 0.1 ml/100 g), two hours before CLP or sham surgery. The results show the mean ± S.E.M of 3–4 experiments with samples from different animals per group. $^{*}P < 0.05$ *versus* control (sham-operated) group, and $\#P < 0.05$ when compared with the CLP-vehicle group.

aortas from rats subjected to CLP, compared with the sham-operated group (Fig. 5B). Importantly, the treatment with doxycycline or ONO-4817 did not change the detected levels of calponin-1 in the sham-operated groups, but completely abrogated the CLP-induced reduction in this protein (Fig. 5B; $P=0.0707$ for interaction factor between sepsis and MMP inhibitors).

4. Discussion

The major novelty of this study is that the oral administration of MMP inhibitors, either doxycycline or ONO-4817, was able to prevent sepsis-associated hyporeactivity to vasoconstrictors in vessels from animals subjected to the polymicrobial CLP model of sepsis.

Although MMPs have been previously associated with vascular dysfunction in endotoxemic rats, the data presented herein reinforces the importance of MMPs or its associated targets in the pathogenesis of vascular refractoriness to pressor agents in septic shock, mainly because the CLP model induces a more aggressive inflammatory scenario and resembles the complexity of human sepsis, while the LPS-model is often subject to criticism, since it does not reproduce the intensity and progression of infection-induced septic shock in humans (for review see Dejager et al., 2011).

Doxycycline, the only MMP inhibitor approved for use in humans (Caton and Ryan, 2011), is a member of the tetracycline antibiotics and has been described as anti-inflammatory in experimental models (Castro et al., 2011; Yrjanheikki et al., 1998). Although we have not conducted experiments to evaluate its potential antibacterial and anti-inflammatory effects, administration

of doxycycline did not improve any hematological parameter commonly associated with sepsis in the CLP group, such as leukopenia and thrombocytopenia, among others (Table 2), suggesting that the low dose of doxycycline adopted in our study, given as a single administration, did not present significant antimicrobial effects. ONO-4817 – also classified as a broad-spectrum MMP inhibitor, but without effects on the activity of MMP-1 and MMP-7 – acts similarly to doxycycline and has also been known for its anti-inflammatory properties (Golub et al., 1998; Naito et al., 2004; Yamada et al., 2000). Importantly, neither doxycycline nor ONO-4817 had any influence on the increased serum levels of NO_x^- detected in CLP rats. Accumulation of NO_x^- in experimental sepsis occurs due the high levels of nitric oxide released mainly by the inducible isoform of the nitric oxide synthase, which in turn has its expression stimulated by inflammatory cytokines produced in response to infection (for review see Dinarello, 1997). Thus, the unaltered serum NO_x^- levels in septic rats treated with doxycycline or ONO-4817 suggest that these drugs did not influence the inflammatory profile associated with the CLP model of sepsis, but instead improved the vascular reactivity to vasoconstrictors by their direct MMP inhibitory activity in vessels.

Previous evidence involving MMPs in the hyporeactivity to vasoconstrictors in sepsis were restrict to the reduced vascular responses to the α1-adrenergic agonist phenylephrine in the LPS model. Importantly, the results obtained in this study not only support these findings, but also disclosed the involvement of MMP activity in reduced vasoconstriction to angiotensin II in aorta from rats subjected to CLP. Low expression levels of both angiotensin II and angiotensin converting enzyme have been suggested as an indicative of poor prognosis in septic patients (Zhang et al., 2014). In addition, a reduced expression of vascular angiotensin II AT_1 receptor subtype has been suggested as a crucial event in experimental sepsis-induced hypotension (Bucher et al., 2001; Mederle et al., 2013). Taking into account the physiological vascular effects and the severe hyporeactivity to angiotensin II found in aortic rings from CLP rats, as well as the protection promoted by either doxycycline or ONO-4817 (Fig. 1D), another important finding of our study is that MMP activity contributes to vascular hyporeactivity to diverse contractile agonizts potentially involved in sepsis-induced vascular failure and severe hypotension. Notably, vessels from sham-operated animals treated with MMP inhibitors did not present any change in their reactivity to vasoconstrictors when compared with control preparations (Fig. 1A and C), disclosing that the ability of both doxycycline and ONO-4817 to increase the vascular reactivity to phenylephrine or angiotensin II was restrict to aortic rings from septic animals, as evidenced by the significant interaction factor between sepsis and MMP-inhibitors found in our statistical analysis.

A number of metalloproteinases, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, and MMP-15, have been involved in different vascular functions in both healthy and disease states (for review see Chen et al., 2013; Galis and Khatri, 2002). Nevertheless, most of the vascular MMPs are described as interstitial collagenases (i.e. MMP-1 and MMP-13), or are found mainly in macrophages or other blood cells after their migration to vessels (i.e. MMP-3, MMP-12 and MMP-13). This study was designed to explore the influence of intracellular MMPs (i.e. MMP-2) on the vascular responses to vasoconstrictors in the CLP model of sepsis, but taking into account the poor selectivity of the MMP inhibitors currently available, one important limitation of our study is that we were unable to discard the individual participation of each MMP in the contractile function in our experiments.

Zymography and Western blot assays were carried out to better explore and confirm the correlation between the improved vascular reactivity to vasoconstrictors and the inhibition of MMP-2 by doxycycline and ONO-4817 in aortas from rats subjected to CLP. As previously demonstrated for the LPS model (Lalu et al., 2006), we found an augmented activity of the 72 kDa MMP-2 in vessels from untreated CLP rats, which was prevented by doxycycline or ONO-4817, and was not dependent on increased expression of MMP-2 protein, as detected by Western blot. MMPs can interact with several structural and functional proteins involved in a number of cellular functions. Their protective or deleterious participation in cardiovascular diseases, including myocardial infarction, heart failure, atherosclerosis, hypertension, stroke, and others, have been extensively studied (for review see Ali et al., 2011; Castro et al., 2011; Papazafiropoulou and Tentolouris, 2009). Nevertheless, neither the mechanisms involved in their activation nor the cellular targets of MMPs are completely understood. Tissue inhibitors of metalloproteinases (TIMPs) are recognized as the main responsible for regulation of MMP activity in tissues, and the reduction of these endogenous protease inhibitors could explain the increased activity of MMP-2 found in our experiments. Notably, this hypothesis can be discarded based in the unaltered levels of TIMP-4 (data not shown) and the increased levels of TIMP-1 (Fig. 4B) detected in aorta homogenates from CLP rats, which did not present a different profile in samples taken from animals treated with MMP inhibitors. Although this study did not provide additional insights regarding the mechanisms involved in MMP activation, it is well known that sepsis is characterized by increased biosynthesis of reactive oxygen- and nitrogen-species, such as nitric oxide, superoxide and peroxynitrite (Rees et al., 1990; Seija et al., 2012). Interestingly, it has been demonstrated that peroxynitrite can activate 72 kDa MMP-2 (Viappiani et al., 2009). Thus, considering the increased gelatinolytic activity found for 72 kDa MMP-2 and the lack of detectable 64 kDa MMP-2 in our experiments, it is reasonable to speculate that oxidative stress may play an important role in the stimulation of vascular MMP-2 in polymicrobial sepsis. However, the involvement of reactive oxygen and nitrogen species and other mechanisms associated with the vascular activation of MMPs in sepsis deserves further investigation.

In spite of the description of a large number of predicted substrates for MMP-2 in the vasculature (Chow et al., 2007), at least to our knowledge only two regulatory proteins of vascular contraction, calponin-1 and smoothelin-B (Rensen et al., 2008; Takahashi et al., 1988), had their role and interaction with MMPs in sepsis previously investigated. Importantly, calponin-1, but not smoothelin-B, was characterized as a target of MMP-2 proteolysis in the LPS model (Castro et al., 2012; de Souza et al., 2014). The lack of changes in the levels of smoothelin-B and the significant reduction in the levels of calponin-1 found in aortas from CLP rats, a finding fully prevented by both doxycycline and ONO-4817, confirmed the relationship between the enhanced activity of MMP-2 and calponin-1 also in the CLP model of sepsis.

5. Conclusions

In summary, this study demonstrates, for the first time in the CLP model of sepsis, that polymicrobial sepsis is associated with increased activity of MMP-2, augmented levels TIMP-1, and reduced amounts of calponin-1 in the aorta of rats. Importantly, all these changes were prevented by doxycycline or ONO-4817, which also protected against sepsis-induced vascular hyporeactivity to both phenylephrine and angiotensin II, confirming the role of MMPs in the genesis of the vascular failure associated with septic shock. These findings reinforce the importance of additional studies to evaluate the participation of MMPs in the functionality of other vascular beds, including resistance vessels, as well as their influence on vascular tone and tissue perfusion in later periods of sepsis. The improved knowledge regarding the beneficial or

detrimental effects of MMP activity in the vasculature during sepsis may provide better strategies in the future management of vascular failure in patients with septic shock.

Conflicts of interest

The authors declare that they have no conflict of interest.

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